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Ca^{2+}-mobilising properties of synthetic fluoro-analogues of *myo*-inositol 1,4,5-trisphosphate and their interaction with *myo*-inositol 1,4,5-trisphosphate 3-kinase and 5-phosphatase

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The ability of two fluoro-analogues of D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) to mobilize intracellular Ca^{2+} stores in SH-SY5Y neuroblastoma cells has been investigated. DL-2-deoxy-2-fluoro-*scyllo*-$Ins(1,4,5)P_3$ (2F-$Ins(1,4,5)P_3$) and DL-2,2-difluoro-2-deoxy-*myo*-$Ins(1,4,5)P_3$ (2,2-F_2-$Ins(1,4,5)P_3$) were full agonists (EC_{50}s 0.77 and 0.41 μM respectively) and slightly less potent than D-$Ins(1,4,5)P_3$ (EC_{50} 0.13 μM), indicating that the axial 2-hydroxyl group of $Ins(1,4,5)P_3$ is relatively unimportant in receptor binding and stimulation of Ca^{2+} release. Both analogues mobilized Ca^{2+} with broadly similar kinetics and were substrates for $Ins(1,4,5)P_3$ 3-kinase but, qualitatively, were slightly poorer than $Ins(1,4,5)P_3$. 2F-$Ins(1,4,5)P_3$ was a weak substrate for $Ins(1,4,5)P_3$ 5-phosphatase but 2,2-F_2-$Ins(1,4,5)P_3$ was apparently not hydrolysed by this enzyme, although it inhibited its activity potently ($K_i = 26$ μM).

Second messenger; Inositol phosphate; Fluoro-analogue; Ca^{2+} mobilisation

1. INTRODUCTION

D-*myo*-inositol 1,4,5-trisphosphate (Fig. 1, (1)) is a second messenger which mediates the release of Ca^{2+} from intracellular stores [1,2] via a receptor which has been cloned and sequenced [3] and which, when reconstituted, mediates Ca^{2+} release in response to $Ins(1,4,5)P_3$ [4]. A major challenge is now the elucidation of the structural basis for interaction of $Ins(1,4,5)P_3$ both with its receptor and with the metabolic enzymes, $Ins(1,4,5)P_3$ 3-kinase and 5-phosphatase, and the rational chemical design of agonists, antagonists and enzyme inhibitors. Recent progress in inositol phosphate chemistry [5,6] and molecular recognition has been reviewed [7].

Several inositol ring-modified and phosphate-modified analogues have been synthesized [5,6] and some progress has been made to understand the role of the 3 phosphate and hydroxyl groups of $Ins(1,4,5)P_3$ in receptor binding specificity and stimulation. Isosteric replacement of a hydroxyl group with fluorine [8] has led to fluorinated *myo*-inositol [9,10] and *myo*-inositol phosphate analogues [11,12]. D-3-fluoro-3-deoxy-*myo*-inositol inhibits cell growth in NIH 3T3 cells [9] and 5-fluoro-5-deoxy-*myo*-inositol is taken up by L1210 cells and incorporated into cellular phospholipid [10]. Three reports of biological activity for ring-modified analogues of $Ins(1,4,5)P_3$, including DL-2-deoxy-$Ins(1,4,5)P_3$ (Fig. 1 (2)), have appeared [13,14,15], but no biological evaluation of fluorinated inositol phosphates has yet been reported.

We report here a study of the interaction of the two synthetic analogues 2-deoxy-2-fluoro-*scyllo*-inositol 1,4,5-trisphosphate (2-F-$Ins(1,4,5)P_3$) (Fig. 1, (3)) and 2,2-difluoro-2-deoxy-*myo*-inositol 1,4,5-trisphosphate (2,2-F_2-$Ins(1,4,5)P_3$) (Fig. 1, (4)) with the Ca^{2+}-releasing receptor of SH-SY5Y neuroblastoma cells and the metabolic enzymes $Ins(1,4,5)P_3$ 5-phosphatase and 3-kinase.

2. MATERIALS AND METHODS

2.1. *Synthetic analogues*

DL-2-deoxy-2-fluoro-*scyllo*-inositol 1,4,5-trisphosphate (Fig. 1, (3)) and DL-2,2-difluoro-2-deoxy-*myo*-inositol 1,4,5-trisphosphate (Fig. 1, (4)) were synthesized from protected *myo*-inositol precursors by respective diethylaminosulphur trifluoride fluorination of 1-prop-1-enyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inositol [16] and 1-allyl-3,6-di-*O*-benzyl-4,5-*O*-isopropylidene-2-*myo*-inosose [16], removal of non-benzylic protecting groups, followed by bis(2-cyanoethyl)-*N,N*-diisopropylamino-phosphine phosphitylation, oxidation of the resulting trisphosphites with *t*-butyl hydroperoxide and deprotection using sodium in liquid ammonia, as described for $Ins(1,4,5)P_3$ [17]. Analogues were purified on an ion-exchange column of DEAE Sephadex A-25 using a gradient of triethylammonium bicarbonate buffers, pH 8.0, and were isolated as the triethylammonium salt and assayed by quantitative phosphate analysis. Full chemical details of the synthetic procedures will appear elsewhere.

2.2. *Cell culture, permeabilisation and Ca^{2+} release*

Monolayers of SH-SY5Y human neuroblastoma cells (passage 75-95), initially a kind gift of Dr J.L. Biedler (Sloane-Kettering In-

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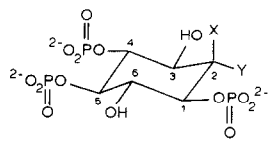

X = OH ; Y = H (1)

X = Y = H (2)

X = H ; Y = F (3)

X = Y = F (4)

Fig. 1. Structures of synthetic analogues of Ins(1,4,5)P_3. Only D-isomers are shown.

stitute, New York, USA), were grown as described [18]. Prior to permeabilisation, the cells were harvested in 10 mM Hepes, 0.9% NaCl pH 7.4, containing 0.02% EDTA, electroporated [20] and $^{45}Ca^{2+}$ release experiments were performed as described [19]. The temporal characteristics of Ca^{2+} mobilisation from electroporated SH-SY5Y cells, maintained in the same buffer as for $^{45}Ca^{2+}$ experiments, were monitored using a Ca^{2+}-specific electrode [21]. All cell culture reagents were from GIBCO Ltd, D-Ins(1,4,5)P_3 was from Calbiochem, $^{45}CaCl_2$ (approx. 1000 Ci/mmol) was obtained from Amersham plc, ATP, quin-2 and oligomycin were obtained from Sigma. EC_{50} values were derived using ALLFIT computer-assisted curve fitting [22]. Combined data from a number of independent experiments (n) are expressed as mean ± SEM, where $n \geqslant 3$.

2.3. *Ins(1,4,5)P_3 5-phosphatase activity*

Human erythrocyte ghosts (7 mg protein/ml) were prepared according to Downes et al. [23] and stored at −40°C. Protein concentration was determined as described [25]. Ins(1,4,5)P_3 or fluoro-analogues (100 μM) were incubated at 37°C for 60 min in the presence of ghosts (5 mg protein/ml) or inactivated (boiled) ghosts in a buffer consisting of 30 mM Hepes, 2 mM $MgCl_2$, pH 7.2. Incubations were terminated by boiling and Ins(1,4,5)P_3 and its analogues were then assayed for ability to release Ca^{2+} from permeabilised SH-SY5Y cells. Inhibition of 5[^{32}P]-Ins(1,4,5)P_3 metabolism by 2,2-F_2-Ins(1,4,5)P_3 was performed essentially as described for inositol 1,4,5-trisphosphorothioate [24]. Erythrocyte ghosts (1 mg protein/ml) were incubated at 37°C for 12 min in the presence of 30 μM Ins(1,4,5)P_3 (approx. 5000 dpm [^{32}P]-Ins(1,4,5)P_3) and 1–100 μM 2,2-F_2-Ins(1,4,5)P_3. Under these conditions no more than 20% of the substrate was consumed.

2.4. *Ins(1,4,5)P_3 3-kinase activity*

Whole rat brains were minced, homogenised, diluted to a concentration of 20% w/v in 150 mM sucrose and centrifuged at 100 000 × g for 90 min. The resulting supernatant was stored at −40°C until required. Ins(1,4,5)P_3 or fluoro-analogues (1 mM) were incubated at 37°C for 60 min in the presence of this crude 3-kinase preparation (5% w/v) or inactivated (boiled) enzyme preparation in a buffer consisting of 50 mM Tris-maleate, 20 mM $MgCl_2$, 10 mM Na_2ATP, 5 mM 2,3-bisphosphoglycerate, 0.1% BSA, pH 7.5. Incubations were terminated by boiling and the mixtures were assayed for ability to release $^{45}Ca^{2+}$ from SH-SY5Y cells.

3. RESULTS AND DISCUSSION

Myo-inositol possesses 5 equatorial hydroxyl groups and one axial hydroxyl group. Using fluorinated Ins(1,4,5)P_3 analogues we have attempted to probe the role of the unique axial 2-hydroxyl group in determining the affinity and specificity of Ins(1,4,5)P_3 for its receptor and metabolic enzymes. The EC_{50} of

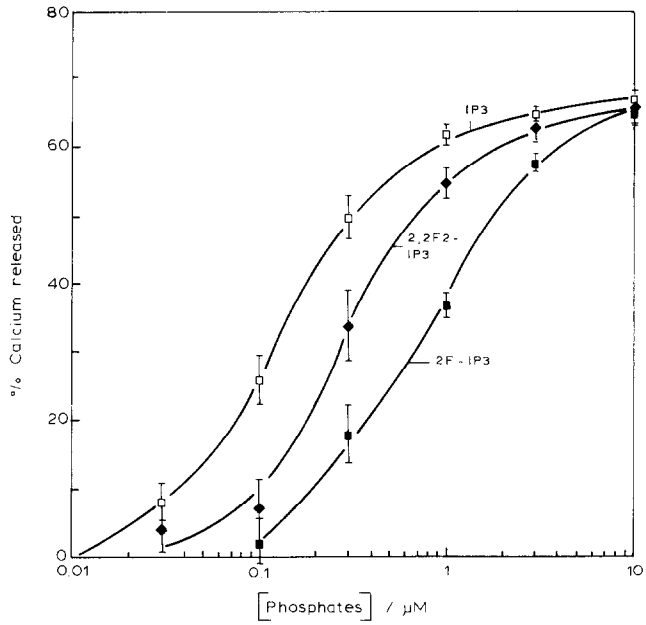

Fig. 2. Dose-response curves for release of Ca^{2+} by Ins(1,4,5)P_3 and analogues in SH-SY5Y neuroblastoma cells. For experimental details see section 2.

Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilised SH-SY5Y human neuroblastoma cells was 0.13 μM (Fig. 2, Table I). For DL-2,2-F_2-Ins(1,4,5)P_3 (Fig. 1, (4)) and DL-2F-Ins(1,4,5)P_3 (Fig. 1, (3)) the EC_{50} values were 0.41 and 0.77 μM respectively (Fig. 2, Table I). Both analogues were full agonists for Ca^{2+} mobilisation (Fig. 2) and released Ca^{2+} with kinetics similar to Ins(1,4,5)P_3, although re-uptake of Ca^{2+} was slower for the analogues than for Ins(1,4,5)P_3 (Fig. 3).

These data suggest that the analogues have slightly lower affinity than Ins(1,4,5)P_3 for the receptor. This has also been confirmed by radioligand binding to the rat cerebellar Ins(1,4,5)P_3 receptor (A.L. Willcocks, S.R. Nahorski and B.V.L. Potter, data not shown). It should be noted, however, that the synthetic fluoro-compounds are racemic mixtures. L-Ins(1,4,5)P_3 has previously been shown to be essentially inactive at binding to and inducing Ca^{2+} release at the stereospecific

Table I

	Ca^{2+} release[1]	Fold shifts in EC_{50}[4]	
	EC_{50} (μM) (n = 12)	3-kinase treatment[2] (n = 3)	5-phosphatase treatment[3] (n = 3–4)
D-Ins(1,4,5)P_3	0.13 ± 0.01	24 ± 6.5	10 ± 3.4
DL-2F-Ins(1,4,5)P_3	0.77 ± 0.08	1.5 ± 0.9	1.2 ± 0.3
DL-2,2-F_2-Ins(1,4,5)P_3	0.41 ± 0.05	7 ± 3.0	0.2 ± 0.3

[1] $^{45}Ca^{2+}$ release from permeabilised SH-SY5Y neuroblastoma cells

[2] After 1 h treatment of Ins(1,4,5)P_3 or analogue (1 mM) with 5% 3-kinase preparation

[3] After 1 h treatment of Ins(1,4,5)P_3 or analogue (100 μM) with 5 mg protein/ml 5-phosphatase at 37°C

[4] Fold shifd is defined by ([EC_{50}(after)/EC_{50}(before)] -1)

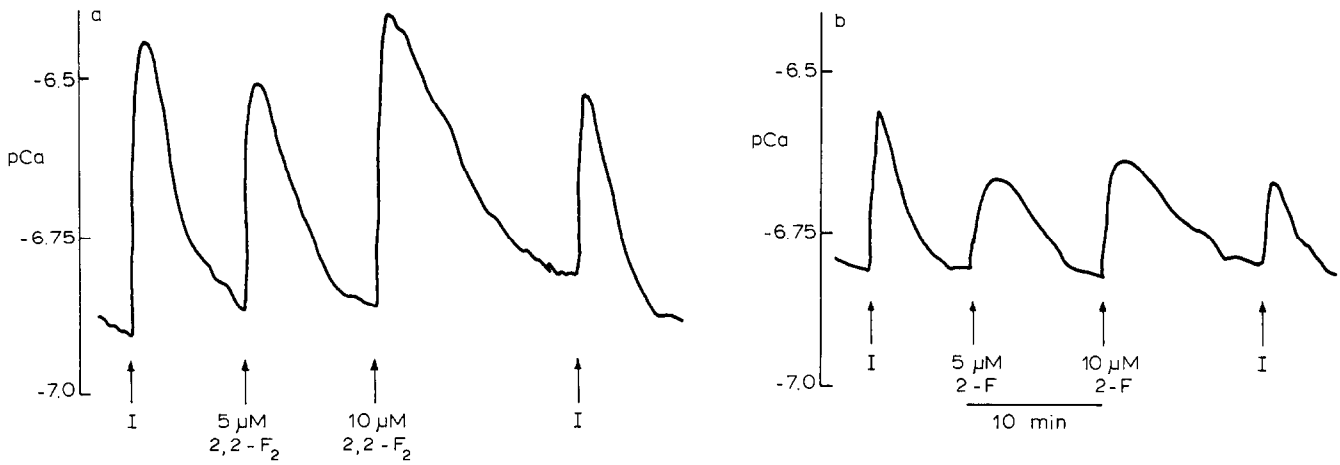

Fig. 3. Kinetics of Ca^{2+} release induced by Ins(1,4,5)P_3 and synthetic fluoro-analogues monitored in electrically permeabilised SH-SY5Y neuroblastoma cells using a Ca^{2+}-specific electrode. Suspensions of permeabilised cells (3–4 mg protein/ml) were challenged with 5 μM D-Ins(1,4,5)P_3 (I) and (a) 5 μM and 10 μM 2,2-F_2-Ins(1,4,5)P_3 or (b) 5 μM and 10 μM 2F-Ins(1,4,5)P_3. Data shown are representative of results from 3 similar independent experiments.

Ins(1,4,5)P_3 receptor [7]. Thus, the true EC_{50} values for D-2,2-F_2-Ins(1,4,5)P_3 and D-2F-Ins(1,4,5)P_3 may be taken as 0.21 μM and 0.39 μM respectively and fluoro-substitution at the 2-position therefore makes the analogues only some 1.6- and 3-fold less potent than Ins(1,4,5)P_3.

These data should be compared with an EC_{50} of 6 μM for D-6-deoxy-Ins(1,4,5)P_3-induced Ca^{2+} release in SH-SY5Y cells (J. Strupish, S. Safrany, D. Dubreuil, S.D. Gero, S.R. Nahorski and B.V.L. Potter, unpublished observations) and with an EC_{50} of 0.5 μM reported for DL-2-deoxy-Ins(1,4,5)P_3-induced Ca^{2+} release (i.e. 0.25 μM for the D-isomer compared with 0.2 μM for D-Ins(1,4,5)P_3) in permeabilised macrophages [14]. Qualitatively, therefore, there is little difference between the 2-deoxy and 2-fluorinated analogues and since the EC_{50} values are only slightly higher than Ins(1,4,5)P_3 itself, the axial 2-hydroxyl, in contrast to the 6-hydroxyl group, cannot be making a major contribution to receptor binding and activation. This is also borne out by the tolerance of the Ca^{2+} mobilising receptor for certain bulky substitutions at the 2-position [14,15].

The three phosphate groups of Ins(1,4,5)P_3 will most likely make ionic interactions with positively charged centres of the Ins(1,4,5)P_3 receptor and make the major contribution to binding energy. The three hydroxyl groups at the 2, 3, and 6 positions will be either hydrogen bond donors to the protein, or acceptors of hydrogen bonds from it. Complete deletion of single or multiple hydroxyl groups can lead to drastic loss of Ca^{2+}-mobilising ability, exemplified by the low potency of D-6-deoxy-Ins(1,4,5)P_3 and DL-1,2,4-cyclohexane trisphosphate [13], but apparently not for 2-deoxy-Ins(1,4,5)P_3 (Fig. 1, (2)) [14]. Isosteric and isoelectronic replacement of a hydroxyl group by fluorine [8], however, might be predicted to have a less drastic effect, provided H-bonding interactions could still be made with the protein. 2,2-F_2-Ins(1,4,5)P_3 and 2F-Ins(1,4,5)P_3 differ structurally only by the fact that the former possesses an axial fluorine atom rather than a proton and is therefore unable to donate a hydrogen bond. 2,2-F_2-Ins(1,4,5)P_3 is clearly a more potent analogue than 2F-Ins(1,4,5)P_3, presumably since the axial fluorine atom is able to make a more favourable interaction with the receptor and, indeed, this analogue is only marginally (1.6-fold) less potent than Ins(1,4,5)P_3 itself. We propose, therefore, that the 2-hydroxyl group of Ins(1,4,5)P_3 accepts a hydrogen bond from the receptor

Ins(1,4,5)P_3 is metabolised by two major routes: phosphorylation by a 3-kinase and dephosphorylation by a 5-phosphatase [7]. Treatment of solutions of Ins(1,4,5)P_3 or fluoro-analogues with a 3-kinase preparation, followed by assay of Ca^{2+}-releasing ability, gave shifts in EC_{50} on account of 3-position phosphorylation as shown (Table I). Clearly, both fluoro-analogues are weaker substrates than Ins(1,4,5)P_3 for the 3-kinase, but 2,2-F_2-Ins(1,4,5)P_3 appeared to be significantly better than 2F-Ins(1,4,5)P_3, presumably since the axial fluorine atom of the former acts more successfully to mimic the normal electronic environment at the 2-position adjacent to the crucial 3-hydroxyl group. Whilst not an absolute requirement for activity, the 2-hydroxyl group is clearly an important element in the recognition of substrates by Ins(1,4,5)P_3 3-kinase.

We have also investigated the interaction of the fluoro-analogues with Ins(1,4,5)P_3 5-phosphatase from human erythrocyte ghosts. Treatment of solutions of Ins(1,4,5)P_3 or fluoro-analogues with a 5-phosphatase preparation, followed by assay of Ca^{2+}-releasing ability, gave shifts in EC_{50}, on account of dephosphorylation as shown (Table I). Thus, whilst 2F-Ins(1,4,5)P_3

appears to be a weaker substrate for 5-phosphatase than Ins(1,4,5)P_3, there was no significant shift in EC_{50} value for 2,2-F_2-Ins(1,4,5)P_3. This is surprising, on account of the known low specificity of 5-phosphatase for substrates [7,13]. Moreover, a study of 2,2-F_2-Ins(1,4,5)P_3-induced inhibition of 5[^{32}P]Ins(1,4,5)P_3 breakdown by erythrocyte 5-phosphatase (data not shown; method similar to [24]) showed that this analogue binds to the enzyme with high affinity ($K_i = 26 \pm 4.1$ μM; cf. K_m for D-Ins(1,4,5)P_3, 40 μM [24]). While Ins(1,4,5)P_3 5-phosphatase is specific for the D-enantiomer of Ins(1,4,5)P_3, the analogue is a racemate and this unexpected result may indicate that L-2,2-F_2-Ins(1,4,5)P_3 has a considerably higher affinity for 5-phosphatase than would be expected (cf. K_i for L-Ins(1,4,5)P_3, 124 μM [24]) and is inhibiting breakdown of D-2,2-F_2-Ins(1,4,5)P_3. This seems plausible in the light of data showing that a 2-arylacyl-substituted L-Ins(1,4,5)P_3 analogue was a highly potent inhibitor of 5-phosphatase with a K_i of 3.8 μM [15]. Further analysis must await resolution of the pure enantiomers of 2,2-F_2-Ins(1,4,5)P_3. The slow hydrolysis of 2F-Ins(1,4,5)P_3 is also in accord with the observation that 2-deoxy-Ins(1,4,5)P_3 is hydrolysed more slowly than Ins(1,4,5)P_3 by both erythrocyte ghost and brain cytosol 5-phosphatase [14]. Our results support the notion that the 2-hydroxyl group of Ins(1,4,5)P_3 is not absolutely required for activity, but it may be involved in substrate recognition by Ins(1,4,5)P_3 5-phosphatase.

The metabolism of the fluoro-analogues was examined using electrically-permeabilised SH-SY5Y cells, which exhibit both Ins(1,4,5)P_3 5-phosphatase and 3-kinase activities [26]. 2,2-F_2-Ins(1,4,5)P_3 and 2F-Ins(1,4,5)P_3 gave similar Ca^{2+}-release profiles to that induced by Ins(1,4,5)P_3, but Ca^{2+} re-uptake, which parallels inositol phosphate metabolism, was retarded (fig. 3); e.g. for 5 μM D-Ins(1,4,5)P_3 the time taken for Ca^{2+} concentration to decay to 50% of the maximal response was on average 2.8 min, whereas for 10 μM DL-2,2-F_2-Ins(1,4,5)P_3 the time taken was 5.4 min. This is consistent with a combination of the effects of 5-phosphatase and 3-kinase already demonstrated.

We conclude that both analogues are recognized by all three Ins(1,4,5)P_3 binding proteins and that the 2-hydroxyl group of Ins(1,4,5)P_3 does not play an absolute role either in receptor binding and activation, or enzyme activity, although it may be an important recognition element.

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